

Analysis of Avian Leukosis Virus DNA and RNA in Bursal Tumors: Viral Gene Expression Is Not Required for Maintenance of the Tumor State

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Summary

Each of twelve tumors induced by either Rous-associated virus-1 or -2 (RAV-1 or RAV-2) contained a predominant population of cells with ALV proviruses integrated at common sites, consistent with a clonal origin. Seven of nine RAV-2-induced bursal tumors contained single proviruses, and all seven solitary proviruses had suffered deletions. The detailed structures of four of these proviruses show that major deletions had occurred near or at the 5' ends, spanning sequences potentially important in the production of viral RNA. One provirus also lacked most of the information coding for the replicative functions of the virus. Restriction maps suggest that these four proviruses were inserted in similar regions of the host genome. We have studied virus-specific RNA in four bursal tumors and four cell lines derived from bursal tumors. No normal viral RNA species were detectable in three tumors containing single aberrant proviruses. However, transcripts of 2.2 kb which reacted only with a hybridization probe specific for the 5' end of viral RNA were observed in one of these three tumors. Analogous species, varying in length from 1.5 to 6.0 kb, were observed in a fourth bursal tumor with multiple proviruses and in all four cell lines. (This tumor and the cell lines also contained normal species of ALV mRNA and apparently normal proviral DNA.) The structures of the aberrant proviruses and the absence of normal viral RNA in some tumors indicate that expression of viral genes is not required for maintenance of the tumor phenotype. In at least some cases, the mechanism of oncogenesis may involve stimulation of transcription of flanking cellular sequences by a viral promoter.

Introduction

Avian leukosis viruses (ALVs) most frequently induce lymphatic neoplasms of the B cell lineage arising in the Bursa of Fabricius of chickens (for review see Purchase and Burmeister, 1978). The characteristics of oncogenesis by ALVs place these viruses in a unique category of avian retroviruses. ALV-induced bursal lymphomas become microscopically evident

only 4-6 weeks after infection and require 4-6 months to reach macroscopic size. ALVs do not transform cells in culture, and no gene responsible for their oncogenic effects has been identified. In contrast, the other major classes of avian retroviruses, the sarcoma and the defective leukemia viruses, induce neoplasms which become grossly apparent within a few weeks after infection and kill the chicken within 1-2 months; in addition, these viruses transform their respective target cells in culture. Rous sarcoma virus has been shown to exert its oncogenic effects via a protein, pp60^{src} (Brugge and Erikson, 1977; Purchio et al., 1978), which is not required for virus replication and is encoded in a gene (*src*) which has been transduced from the cellular genome (Stehelin et al., 1976; Spector et al., 1978a). Transduced host cell sequences also appear to be responsible for the oncogenic effects of the defective leukemia viruses and other sarcoma viruses (Sheiness and Bishop, 1979; Roussel et al., 1979).

The mechanism by which ALVs might exert their oncogenic effects has remained obscure. ALVs do not appear to contain transduced cellular sequences or to encode proteins other than those required for replication (Vogt, 1977). Sequences located near the 3' end of viral RNA have been implicated in ALV oncogenesis (Tsichlis and Coffin, 1980) because this region exhibits the only major divergence (Neiman et al., 1977; Coffin et al., 1978; Shank et al., 1981) from the sequences of a nononcogenic avian retrovirus endogenous to some normal chickens, RAV-0 (Motta et al., 1975; Purchase et al., 1977).

We have begun to investigate the mechanism of ALV-induced tumorigenesis by analyzing the ALV-specific DNA and RNA found in bursal lymphomas and tumor cell lines derived from ALV-induced bursal lymphomas. We have found that the tumors appear to be clonal and that several contain single proviruses, allowing us to determine the structure of the provirus presumably responsible for oncogenesis in each case. Most of these proviruses exhibit deletions which span regions potentially important in viral RNA biogenesis. At least one deletion also removes most of the genetic information present in the provirus.

Our analysis of virus-specific RNA in tumors and tumor cell lines has revealed RNA species which may result from transcription of host cell sequences initiated at viral promoters. In addition, in three tumors with solitary, defective proviruses, we were unable to detect normal viral mRNAs. Similar results have been obtained by Neel et al. (1981).

Results

Restriction Map of RAV-2 DNA

In order to analyze RAV-2 proviruses present in bursal tumors, it was necessary to construct a map of restriction endonuclease recognition sites present in RAV-2

DNA. Using techniques similar to those described by Shank et al. (1978), we derived a physical map of restriction sites in RAV-2 linear DNA. The approximate relationships between restriction sites, viral RNA, viral genes and the long (330 bp) terminal redundancy (LTR) are shown in Figure 1. Kpn I, Sac I and Hind III cleave the viral DNA once. Eco RI and Bam HI each produces three internal fragments from RAV-2 DNA, some of which comigrate with internal fragments from proviruses endogenous to chickens used in our study. However, the 2.3 kb and 1.1 kb Eco RI fragments and the 1.8 kb Bam HI fragments are derived only from exogenous RAV-2 proviruses and have been used as signature fragments diagnostic of specific regions of the RAV-2 provirus (see Figure 1).

ALV-Induced Tumors Are Clonal and Contain Few ALV Proviruses

In this report we present results obtained with tumors from eight SPAFAS animals and one $15I_6 \times 7_2$ animal inoculated with RAV-2. In addition, we have analyzed tumors from three $15I_6 \times 7_2$ birds infected with RAV-1 (see Experimental Procedures). Table 1 summarizes information concerning each of the tumors used in the experiments described in subsequent sections.

Digestion of proviral DNA with restriction endonucleases produces two types of virus-specific fragments: fragments containing viral sequences linked to host sequences (junction fragments) and internal frag-

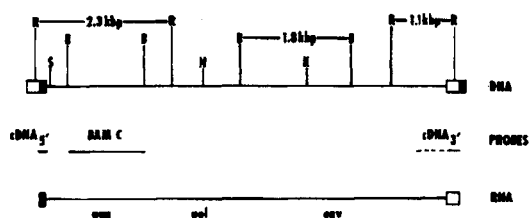


Figure 1. Diagrammatic Representation of Restriction Enzyme Recognition Sites in RAV-2 DNA

The positions of the restriction sites were determined as described by Shank et al. (1978). Unintegrated RAV-2 DNA was prepared from the Hirt supernatant fraction of chicken cells acutely infected by the virus stock also used for induction of tumors in chickens. Restriction fragments were ordered using probes specific for various regions of the viral RNA, sequential digestions with two restriction enzymes and comparison of the restriction fragment patterns of form I and form III viral DNA. These data were supplemented with information obtained from restriction enzyme digests of RAV-2 DNA cloned in bacteriophage λ gtWES- λ B (see Experimental Procedures). R: Eco RI; B: Bam HI; S: Sac I; K: Kpn I; H: Hind III. The terminal repeats (LTR), approximately 300 bp, are drawn as boxes at the ends of the DNA. The open box represents sequences specific to the 3' end of the viral RNA (U_3); the shaded boxes represent sequences specific to the 5' end of viral RNA (U_5). The approximate locations of viral genes are indicated on the diagram of viral RNA. "Signature fragments," which distinguish RAV-2 DNA from proviruses endogenous to the chickens used in this study, are marked by lines connecting the two restriction sites. The sizes of these fragments in kilobase pairs (kbp) are shown. Some of the probes used in our studies represent the regions delineated by the labeled lines between the diagrams of viral RNA and DNA. Descriptions of the content of each probe are included in the Results.

Table 1. Number and Structure of Proviruses in ALV-Induced Tumors

Source	Parental Lines	Chicken Number	Virus Inoculated	Virus Isolated from Bursal Tumor*	Tissues Containing Transformed Lymphocytes†	Number of Exogenous Proviruses	Structure of Exogenous Proviruses	Endogenous Proviral Loci‡
SPAFAS	11	1	RAV-2	—	B	1	Defective	ev1, ev4
SPAFAS	11	2	RAV-2	+	B	1	Defective	ev1, ev4
SPAFAS	11	3	RAV-2	—	B	1	Defective	ev1, ev4
SPAFAS	11	4	RAV-2	—	B	1	Defective	ev1
SPAFAS	11	5	RAV-2	—	B,L	1	Defective	
SPAFAS	11	6	RAV-2	+	B,L,S	1	Defective	
SPAFAS	11	7	RAV-2	—	B,L,S	1	Defective	
SPAFAS	11	11	RAV-2	—	B	2(?)	Defective	ev1
RPRL	$15I_6 \times 7_2$	21	RAV-2	—	B,L,S	3	Defective and nondefective§	
RPRL	$15I_6 \times 7_2$	31	RAV-1	—	B,S	4	Nondefective¶	
RPRL	$15I_6 \times 7_2$	32	RAV-1	—	B,S	3	Nondefective¶	
RPRL	$15I_6 \times 7_2$	33	RAV-1	—	B	3	Nondefective¶	

* See Experimental Procedures.

† B: bursa; L: liver; S: spleen.

‡ ev1 and ev4 are endogenous proviruses associated with the gs^- chl^- phenotype of certain chicken lines and have been defined by restriction endonuclease analyses and breeding experiments (Astrin et al., 1979). In cases for which no loci are listed, the endogenous loci were not characterized.

§ Eco RI digestion of DNA from this tumor produced the expected 2.3 kb and 1.1 kb fragments in addition to other Eco RI fragments containing sequences normally found in the 2.3 kb Eco RI fragment (see text).

¶ Eco RI digestion of DNA from these tumors did not reveal aberrant fragments. This does not preclude the presence of abnormal RAV-2 proviruses (see text).

ments common to all normal proviruses, regardless of the integration site in host cell DNA. Because retroviral DNA can enter many sites in host DNA (Hughes et al., 1978; Steffen and Weinberg, 1978), analysis of junction fragments requires a clonal population of cells, homogeneous with respect to sites occupied by proviral DNA. Studies of proviruses in murine tumors induced after long latency by leukemia and mammary tumor viruses have indicated that such tumors are clonal or semiclonal (Steffen and Weinberg, 1978; Cohen et al., 1979; Cohen and Varmus, 1980; Jahner et al., 1980) and hence amenable to full analysis of proviruses with restriction enzymes. We (see below) and others (Neiman et al., 1980; Neel et al., 1981; Y. K. Fung and H. J. Kung, personal communication) have confirmed this observation using tumors induced by the ALVs.

The presence of ALV-related endogenous proviruses in most chickens complicates the analysis of restriction fragments of ALV DNA in tumors. Hughes et al. (1981b) and Hayward et al. (1979) have constructed physical maps of most of the endogenous proviruses identified by Astrin et al. (1979). We have thus been able to identify the endogenous proviruses present in several of the tumors analyzed (see Table 1).

To address the issue of clonality and to estimate the number of copies of ALV DNA in each tumor, tumor DNAs were initially tested with enzymes which cleave once in RAV-2 proviral DNA. Such enzymes produce two fragments from each normal RAV-2 provirus present in the tumor cells; these fragments are absent from parallel digests of DNA from uninvolved tissue from the same bird. Analyses of DNA from two tumors are illustrated in Figure 2. Hybridization of a probe representing the entire viral genome (cDNA_{rep}) to a Kpn I digest of DNA from tumor LL 5 revealed two fragments (Figure 2 lane 1) not found in a digest of DNA from uninfected normal liver cells (Figure 2 lane 3). This tumor was thus clonal or semiclonal and probably contained only one new exogenous provirus. Annealing of cDNA_{rep} to a Hind III digest of DNA from bursal tumor LL 6 produced two bands (lane 4) which were absent in a digest of DNA from uninfected red blood cells (lane 6). This tumor was clonal and also apparently contained a single RAV-2 provirus.

Provirus in Metastatic Growths Are Identical to Those in the Primary Tumors

Some birds with bursal tumors were found to contain metastases in the spleen or liver. We were thus able to ask whether the metastatic lesions were clonal and whether they contained the same RAV-2 proviruses as the primary tumors.

A Kpn I digest of DNA from a focus of tumor cells present in the liver of chicken 5 (Figure 2 lane 2) was indistinguishable from the digest of bursal tumor DNA (lane 1), suggesting that the metastatic cells also contained a single RAV-2 provirus integrated at the

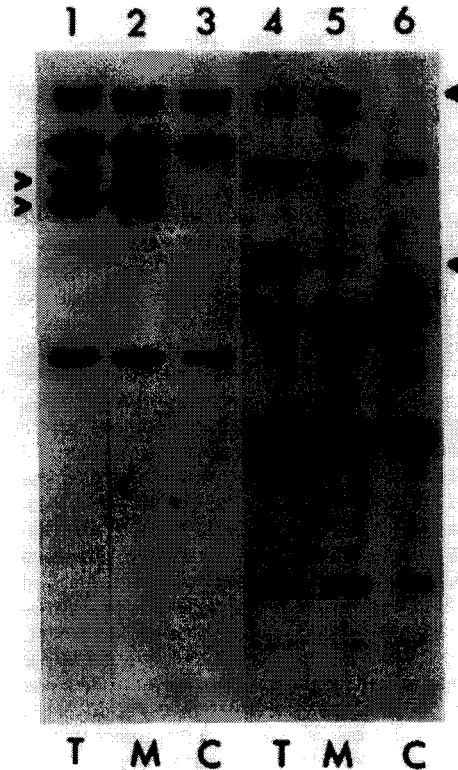


Figure 2. Demonstration of Clonality of Bursal Tumors and Metastases by Digestion of Tumor DNA with Restriction Enzymes Which Cleave Once within the RAV-2 Provirus

Five micrograms of DNA from primary tumors (T), metastatic tumors (M) and control tissues (C) were digested to completion with Kpn I or Hind III, electrophoresed through 0.8% agarose, transferred to nitrocellulose and annealed to cDNA_{rep} as described in Experimental Procedures.

(Lanes 1-3) Kpn I digests of DNA from tumor, hepatic metastasis and normal liver from bird 5. (Lanes 4-6) Hind III digests of DNA from tumor, splenic metastasis and circulating red blood cells from bird 6. The arrows denote the positions of fragments specific to tumor tissue.

same location as the provirus in the bursal tumor cells. The liver tumor apparently resulted from proliferation of bursal tumor cells without amplification or extensive alteration of proviral DNA. These conclusions were supported by further mapping experiments with material from chicken 5, using additional enzymes and hybridization probes (data not shown), and by analysis of DNA from the bursal tumor, a splenic metastasis and uninfected circulating red blood cells from bird 6. As revealed by digestion with Hind III (lanes 4-6), the primary tumor and the metastatic growth appeared to harbor the same single RAV-2 provirus.

The Single RAV-2 Provirus Present in Bursal Tumor LL 1 Is Defective

The existence of clonal tumors containing single exogenous proviruses allowed us to construct physical

maps of the proviruses presumably responsible for tumorigenesis. In Figure 3, we present a partial analysis of the single provirus in tumor LL 1. Kpn I produced two tumor-specific fragments of 11.8 and 8.2 kb from LL 1 DNA (Figure 3A lanes 1 and 2), as expected for a clonal growth bearing a single new provirus. The 11.8 fragment was clearly distinguishable from a similarly sized fragment containing endogenous proviral DNA in an autoradiogram obtained after a shorter exposure. This result was confirmed in tests with Hind III and Sac I, both of which cleave RAV-2 DNA once (data not shown). To investigate the genetic composition of this provirus, we annealed RAV-2 cDNA₃ to the Kpn I digest of LL 1 DNA (Figure 3A lanes 3 and 4). cDNA₃ is complementary to unique sequences located at the 3' end of the viral RNA (U₃; see Figure 1 and Experimental Procedures), and it should anneal to both of the tumor-specific Kpn I fragments by virtue of the U₃ sequences located in the proviral LTRs. However, only the 8.2 kb Kpn I fragment reacted with RAV-2 cDNA₃; the 11.8 kb fragment did not react (Figure 3A lanes 3 and 4). The Bam C probe, specific for sequences located in the gag gene of both exogenous and endogenous proviruses (see Figure 1), annealed to the 11.8 kb Kpn I fragment, but not to the 8.2 kb fragment, identifying the larger fragment as the left junction fragment (Figure 3A lanes 5 and 6). The simplest interpretation of these data is that the RAV-2 provirus present in this tumor lacked U₃ sequences at the left cell/provirus border.

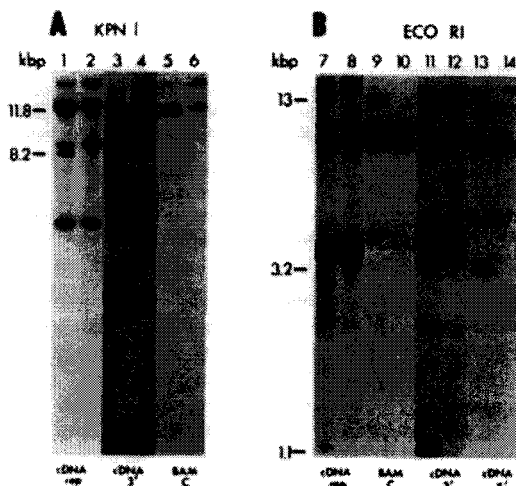


Figure 3. Definition of a Deletion in the Single RAV-2 Provirus in Tumor LL 1

After digestion with Kpn I (A) or Eco RI (B), DNA from bird 1 was analyzed as described in Figure 2. Each set of two lanes shows the results with bursal tumor DNA (even-numbered lanes) and uninvolved spleen DNA (odd-numbered lanes) hybridized to the virus-specific probes indicated below the lanes. The composition of each probe is described in the text and illustrated in Figure 1. Tumor-specific bands are marked with their lengths in kb.

Digestion of DNA from LL 1 with an enzyme, Eco RI, that cleaves RAV-2 proviral DNA at multiple sites, supported this observation. Eco RI digestion of a normal RAV-2 provirus, coextensive with linear viral DNA, should produce three internal fragments (see Figure 1) which can be detected with cDNA_{rep}. Only about 150 bp at each end of proviral DNA remain joined to cell sequences, and the Eco RI junction fragments cannot be detected with cDNA_{rep}. The 3.8 kb internal fragment, bearing sequences from the center of the RAV-2 provirus, comigrates with a similar fragment from the endogenous proviruses present in these chickens. However, the internal restriction fragments of 2.3 and 1.1 kb are unique to the RAV-2 provirus because the Eco RI recognition sites located in the RAV-2 LTRs do not occur in the LTRs of the endogenous proviruses (Hughes et al., 1981b).

Eco RI digestion of the RAV-2 provirus in LL 1 generated the expected 1.1 kb Eco RI fragment, but the 2.3 kb fragment was absent (Figure 3B lanes 7 and 8). (The 1.1 kb fragment is difficult to see in lane 7, but it was clearly evident in the autoradiogram and readily visualized by annealing with cDNA₃ [lane 11].) An additional unexpected Eco RI fragment of 13 kb was detected in the digest of the tumor DNA. The Bam C probe, composed of sequences contained entirely within the normal 2.3 kb Eco RI fragment, hybridized to the 13 kb fragment (lanes 9 and 10). These findings suggest that a deletion which removed the Eco RI site in the U₃ region of the left LTR linked sequences normally found within the 2.3 kb internal fragment to host sequences (see Figure 4).

This interpretation was supported by annealing with cDNA₃, which should detect four Eco RI fragments from a normal RAV-2 provirus: two internal fragments and two host-provirus junction fragments (see Figure 1). If the U₃ sequences in the left LTR have been deleted, then cDNA₃ should react only with the internal 1.1 kb fragment and the right junction fragment. As predicted, cDNA₃ did not react with the 13 kb fragment but did anneal to the 1.1 kb fragment and also to a fragment, presumably the right-hand junction fragment, migrating at 3.2 kb (this band was weak due to the small region of homology [60 bp]) (Figure 3B lanes 11 and 12).

cDNA₅ contains sequences complementary to the bases unique to the 5' end of viral RNA (U₅). These sequences are also located in the LTRs (see Figure 1). Normally cDNA₅ would anneal to the internal 2.3 kb fragment and to the right host-provirus junction fragment in an Eco RI digest of RAV-2 proviral DNA, but cDNA₅ reacted only with the 3.2 kb fragment from the abnormal RAV-2 provirus in LL 1 (lanes 13 and 14). This confirmed the identity of the 3.2 kb fragment as the right host-provirus junction fragment. The failure of either cDNA₃ or cDNA₅ to hybridize to the 13 kb fragment indicated that both U₃ and U₅ sequences were missing from the left LTR (Figure 4).

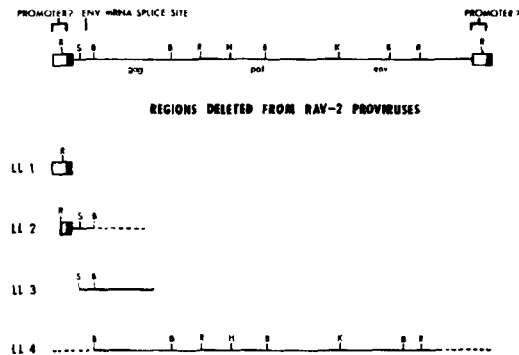


Figure 4. Physical Maps of Regions Deleted from Single Proviruses Present in Four Bursal Tumors

The diagram of RAV-2 DNA (top) illustrates the LTRs, relevant restriction sites and approximate gene locations. The region which may contain the proviral promoter and polyadenylation signals (Shank et al., 1978; Hughes et al., 1978; Sabran et al., 1979; Czernilofsky et al., 1980; Tsichlis and Coffin, 1980; Swanstrom et al., 1981), and the sequences containing the *env* mRNA splice donor site (P. Hackett and G. Gasic, personal communications) are indicated. The region deleted from the provirus in each tumor (LL 1 through LL 4) is drawn below the RAV-2 DNA. These diagrams represent minimal estimates of the extent of each deletion. The deletions could extend into the regions dotted in the diagrams of the proviruses from LL 2 and LL 4. The RAV-2 DNA in LL 4 retained one LTR, but we are uncertain whether it originated at the left or right end of the provirus.

Many Tumors Contain Abnormal Proviruses

The results in the previous section suggest that one of the bursal tumors contained an abnormal provirus, and demonstrate how *Eco* RI can be used to screen tumors for proviruses that have suffered major alterations.

We have analyzed DNA from 12 tumors with *Eco* RI and with at least one enzyme which cleaves once in RAV-2 DNA (data not shown). Seven of these tumors appeared to contain single RAV-2 proviruses (Table 1). All seven solitary proviruses have sustained alterations that affect at least the region defined by the *Eco* RI 2.3 kb fragment. The tumors containing multiple proviruses were more complicated. One tumor probably contained two defective exogenous proviruses; another probably contained both defective and nondefective exogenous proviruses. Finally, DNA from three other tumors yielded all of the expected *Eco* RI fragments but no aberrant fragments. However, this analysis was insufficient to exclude the presence of one or more abnormal proviruses in these tumors.

Physical Maps of Single Aberrant RAV-2 Proviruses in Tumors LL 1-LL 4

We have used the strategies illustrated with LL 1 to construct detailed physical maps of single aberrant RAV-2 proviruses present in four tumors (LL 1-4). These experiments are not shown (data available upon request). In each case, single deletions appeared to

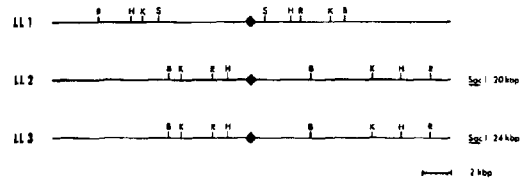


Figure 5. Maps of Restriction Sites in Host Cell DNA Flanking the RAV-2 Provirus in Three Tumors

The sites of integration are marked by diamonds. The restriction enzyme symbols are the same as in Figure 1. The maps are oriented so that transcription of the sense strand of proviral DNA would proceed from left to right (see Figure 1).

account for the mapping data; the extent of the lesions is diagrammed in Figure 4 and discussed more fully below.

Physical Maps of DNA Flanking Proviruses in LL 1, LL 2 and LL 3 Reveal Similar Integration Sites

Using the mapping data from which the proviral deletions were deduced, it was possible to construct physical maps of the regions of chicken DNA which had acquired RAV-2 proviruses in LL 1, LL 2 and LL 3; these maps are depicted in Figure 5.

The restriction enzyme recognition sites in the DNA flanking the proviruses in tumors LL 1 and LL 3 could be unambiguously mapped, based on the sizes of the host-provirus junction fragments. Since the exact size of the deletion in the provirus in tumor LL 2 was not determined, we could not be certain of the absolute distances between the recognition sites encompassing the deletion, although we were able to estimate the distances to within 1 kb (Figure 4). Moreover, the deletion must affect the size of each host-provirus junction fragment similarly; the order and position of the sites could therefore be determined relative to each other on each side of the provirus.

The positions of recognition sites for *Bam* HI, *Kpn* I, *Eco* RI and *Hind* III to the right of the proviruses in tumors LL 2 and LL 3 were identical. The recognition sites of these enzymes to the left of the proviruses had the same order and same position relative to each other. If we assume that the deletion in the provirus of tumor LL 2 spanned 1.2 kb (a reasonable estimate, based upon the data), then the recognition sites were identical on both sides of the integration sites occupied in LL 2 and LL 3. (We have made this assumption in Figure 5.)

Sac I did not cleave either of these proviruses. The *Sac* I fragment containing the provirus in tumor LL 2 was 20 kb, and the provirus-containing *Sac* I fragment from the DNA of tumor LL 3 was 24 kb. The extent of the deletions in the two proviruses could not differ sufficiently to account for this 4 kb difference. Thus there must have been a difference in the position of the *Sac* I recognition sites in the host sequences flanking the proviruses. However, the cell sequences flanking the proviruses in tumors LL 2 and LL 3 seem

to be very similar and might exhibit only minor differences, including one within the 6 bases comprising a Sac I recognition site. Many examples of genetic polymorphism recognized in this fashion have been reported (Mandel et al., 1978; Weinstock et al., 1978; Lai et al., 1979; Hughes et al., 1979).

Comparison of the maps depicted in Figure 5 reveals that the positions of restriction sites in DNA flanking the LL 1 provirus are very similar to the sites in DNA flanking the LL 2 and LL 3 proviruses, but inverted in orientation. (Again Sac I appeared to differentiate the integration sites, but the differences may be due to sequence polymorphisms as described above.) We suggest that similar regions of the host genome have been used as integration sites in LL 1–LL 3, but that the provirus in LL 1 was inserted in an orientation opposite to that of the proviruses in LL 2 and LL 3 (see Discussion).

The limited number of useful restriction sites in the truncated provirus in LL 4 stymied efforts to generate a detailed map of the integration site. However, single and double digestions with Eco RI and Sac I have shown that sites for these enzymes were arranged on both sides of the LL 4 provirus in the same pattern as found for the LL 1 provirus (data not shown). It is thus possible that the integration site in LL 4 is similar or identical to that used in the other three tumors.

Bursal Tumors May Lack Normal RAV-2 mRNAs and Exhibit Provirus-Promoted Transcription of Flanking Cellular DNA

Provirus contains regions which may supply sequences important in initiation, polyadenylation and splicing of viral RNA. The deletions in the solitary proviruses LL 1–LL 4 spanned either the postulated promoter region, the *env* mRNA donor splice site or both (see Figure 4). The deletion in the LL 4 provirus also removed most of the coding information present in the RAV-2 provirus (Figure 4). These results suggested that normal expression of viral genes could not occur in these tumors and that viral gene products may not be necessary for maintenance of the tumor state.

To examine this possibility, we have attempted to analyze viral RNA in bursal tumors, using gel electrophoresis to determine the size and hybridization kinetics to measure the concentration of ALV-related RNA. In Figure 6 we present autoradiograms displaying viral RNA species, detectable with various hybridization reagents, from a tumor bearing multiple RAV-2 proviruses (LL 21) and a tumor bearing a single defective RAV-2 provirus (LL 1). Analysis of RNA from tumor LL 21 showed that all three hybridization probes, cDNA_{rep}, cDNA_{3'} and cDNA_{5'}, detected the normal RAV-2 mRNAs of 8.4 kb (mRNA²⁰⁰ and mRNA^{200-P0}) and 3.2 kb (mRNA^{env}) (Figure 6 lanes 1–3) expected in RAV-2-infected cells (Hayward, 1977; Weiss et al., 1977; Lee et al., 1979; Quintrell et al.,

1980); these species were also observed in parallel analyses of RAV-2-infected fibroblasts (Figures 7A–7C lane 1). However, cDNA_{5'} also detected an RNA species of about 2.4 kb which failed to anneal with the other cDNAs (lane 3).

LL 1 did not appear to contain the normal RAV-2 RNAs of 8.4 and 3.2 kb (Figure 6 lanes 4–6), as predicted from the structure of the provirus in this tumor (see Figure 4). Again, cDNA_{5'} recorded an RNA species (2.2 kb) (Figure 6 lane 6) which did not anneal to cDNA_{rep} or cDNA_{3'} (lanes 4 and 5). We presume

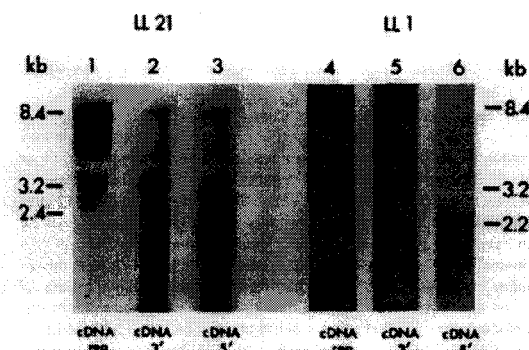


Figure 6. Atypical Species of Viral RNA from a Tumor Harboring Multiple Proviruses and a Tumor Harboring a Single Provirus

Total RNA (80 µg) from tumor LL 21 and tumor LL 1 was electrophoresed through 1.2% agarose containing methyl mercury hydroxide, transferred to activated DBM-cellulose paper and annealed to virus-specific probes as described in Experimental Procedures. Results shown in lanes 2 and 3 and in lanes 4–6 were obtained by annealing different probes sequentially to RNA on the same filter.

(Left-hand panel) total RNA from tumor LL 21; (right-hand panel) RNA from tumor LL 1. The probes employed for annealing in each panel are indicated underneath. The RNAs are marked by arrows and their lengths are indicated in kb.

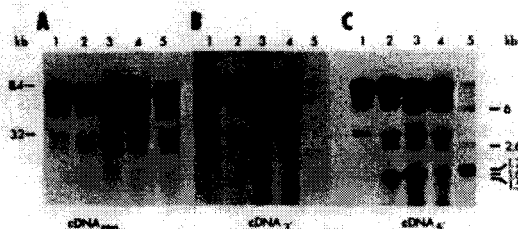


Figure 7. Detection of Atypical Viral RNA Species in Cell Lines Derived from ALV-Induced Bursal Lymphomas

Total RNA (50 µg) from RAV-2-infected fibroblasts and from tumor lines described in the text was electrophoresed through 1.2% agarose containing methyl mercury hydroxide, transferred to activated DBM-cellulose paper and annealed to virus-specific probes as described in Experimental Procedures. The probes are indicated beneath each panel. (Lane 1) RAV-2-infected fibroblast RNA; (lane 2) line BK 4484A RNA; (lane 3) line 1104B-1 RNA; (lane 4) line 1104X-5 RNA; (lane 5) line R2B RNA. The sizes of various RNA species (in kb) detected only with cDNA_{5'} are marked at the right; the sizes of the normal species of RAV-2 mRNA are marked at the left. The RNA on the DBM-cellulose filter was sequentially annealed to each probe as described in Experimental Procedures.

that such transcripts join sequences copied from the U₅ region of RAV-2 DNA to sequences copied from flanking cellular DNA; mechanisms by which such transcripts might be generated are considered in the Discussion. We failed to detect any virus-specific RNA in samples of RNA from tumors LL 2 and 3 analyzed in parallel with RNA from tumor LL 1 using cDNA_{rep}, cDNA_{3'} and cDNA_{5'} (data not shown).

The analysis of gel-fractionated RNA from tumor LL 1 was corroborated by determining the kinetics of hybridization of cDNA_{rep} and cDNA_{5'} to LL 1 RNA in solution (data not shown). Seventy percent of cDNA_{5'} annealed to RNA from tumor LL 1 with a Crt_{1/2} of 9×10^{-3} mole-sec/l. This corresponds to approximately 10 copies of the 2.2 kb RNA per cell. Only 25% of cDNA_{rep} hybridized at a Crt_{1/2} value of 2×10^{-4} mole-sec/l. This low level probably represented annealing of cDNA_{rep} to transcripts from endogenous proviruses and cellular *src* (Wang et al., 1977; Spector et al., 1978b). Parallel tests of RNA from ALV-producing cultured cells (BK 4484A) indicated that the cDNA_{5'} and cDNA_{rep} used in these experiments hybridized at similar rates and to similar extents with normal viral RNA (data not shown). Again, parallel solution hybridization of RNA from tumors LL 2 and LL 3 to cDNA_{5'} and cDNA_{rep} failed to reveal virus-specific RNA present in concentrations higher than that expected from transcripts of endogenous proviruses (data not shown).

Cultured Bursal Tumor Cells Also Contain RNA Species Detected Only with cDNA_{5'}

Three tissue culture lines established by Hihara et al. (1974, 1977) (BK4484A; 1104B-1; 1104X-5) and a fourth (R2B) established by one of us (S. A. Courtneidge) from a tumor passaged in vivo by Okazaki et al. (1980) all contain multiple acquired ALV proviruses (data not shown). Viral RNA species were detected using probes specific for various regions of the viral genome (Figures 7A-7C lanes 2-5). Each line contained the normal two species of viral RNAs (8.4 and 3.2 kb) which reacted with cDNA_{rep}, cDNA_{3'} and cDNA_{5'} (Figures 7A-7C) and were indistinguishable from species in RAV-2-infected fibroblasts (Figures 7A-7C lane 1). However, each tumor line also contained RNA species detected only with cDNA_{5'}; such species were not observed in RAV-2-infected fibroblasts (lane 1 in each panel) or in uninfected tissues from tumor-bearing or normal chickens (data not shown). The BK 4484A cells exhibited two RNA species (2.6 and 1.5 kb) which reacted only with cDNA_{5'} (Figure 7C lane 2). The cell lines 1104B-1 and 1104X-5, which were derived from the same tumor (Hihara et al., 1974) but had slightly different patterns of proviral restriction fragments, contained similar RNA species of 6 kb and 1.6 kb detected only with cDNA_{5'} (Figure 7C lanes 3 and 4). Three size species of RNA (6 kb, 2.6 kb and 1.7 kb) were identified only with cDNA_{5'} in

R2B cells (Figure 7C lane 5).

The most abundant RNA species detected only with cDNA_{5'} in each line was present at approximately 50 to 100 copies per cell, as estimated from the intensity of bands in Figure 7C and from kinetic measurements of total viral RNA in line BK4484A.

Discussion

We have described the physical structure and genetic composition of proviruses and virus-specific RNAs present in bursal lymphomas from chickens with leukemia caused by avian leukosis virus and in tissue culture lines derived from ALV-induced bursal tumors. We have found that all tumors appear to be clonal (that is, each tumor consisted of a predominant population of cells containing at least one provirus integrated at a common site); most (9 of 12) tumors contained proviruses which incurred major deletions detected by restriction enzyme analyses (in 7 of 12 tumors the altered provirus is the only exogenous provirus present in the tumor cells); in at least three tumors no normal species of viral RNA were detectable, suggesting, in conjunction with the aberrant structure of several solitary proviruses, that expression of viral genes is not required for maintenance of the tumor state; some bursal lymphomas and cell lines derived from bursal lymphomas exhibited RNA species which probably consist of sequences from the 5' end of viral RNA joined to cell sequences; and in at least three tumors, single RAV-2 proviruses were located at very similar, if not identical, sites in the host genome. This constellation of findings is consistent with the proposal that ALVs may exert their oncogenic effects by altering expression of a subset of host genes, rather than by elaboration of a viral gene product. Similar data have been set forth by Neel et al. (1981).

Expression of Replication Functions Is Unnecessary for Maintenance of the Tumor State

The detailed physical maps of four solitary proviruses in bursal tumors suggest that normal expression of replication genes could not occur and therefore is not necessary for maintenance of the oncogenic state (Figure 4). Three of the proviruses sustained deletions which affected either the presumed viral promoter or the donor splice site for *env* mRNA. The fourth provirus incurred a deletion which removed most of the genetic information of the provirus. The apparent absence of normal species of viral RNA in the tumors LL 1-LL 3 further supports the idea that expression of replication functions is unnecessary for maintenance of the tumor state. However, we cannot exclude the possibility that viral genes are instrumental in the initiation of tumor growth, since all of our experiments were performed with materials from advanced neoplasms. As expected from the structures of the pro-

viruses in each tumor, we were unable to detect subgroup B virus in the bursal tumors LL 1, LL 3 and LL 4 (see Experimental Procedures). Subgroup B virus was detected in tumor LL 2. We believe this virus was produced by non-tumor cells containing a complete RAV-2 provirus which were present in the bursal tumor at a level undetectable by our hybridization analyses.

Novel Species of RNA Are Present in Some Bursal Tumors and Tumor Cell Lines

Although normal viral RNAs may be absent from tumor cells, we have observed RNA species which anneal only to cDNA_{5'} in tumor cell lines and in some tumors. These findings support the hypothesis that ALV induces tumors by promoting transcription of flanking cellular genes (see below), but we have also encountered two tumors (LL 2 and LL 3) in which no virus-related RNA could be detected. The findings with LL 2 and LL 3 could mean that the hypothesis is incorrect, that a second mechanism is also operative or that late changes in provirus structure or transcriptional activity have obscured the initiating events.

Virus-specific RNAs which react only with cDNA_{5'} were first observed by Quintrell et al. (1980) in lines of ASV-transformed mammalian cells. These transcripts were postulated to consist of viral U₅ sequences joined to host cell sequences. Synthesis of

normal species of viral RNA is thought to be initiated at the site within the left LTR corresponding to the capped nucleotide at the 5' end of the viral genome (Hughes et al., 1978; Sabran et al., 1979; Tsichlis and Coffin, 1980). Putative promoter sequences have been identified in the U₃ region and are thus found at both ends of each normal provirus (Sutcliffe et al., 1980; Van Beveran et al., 1980; Dhar et al., 1980; Shimotohno et al., 1980; Czernilofsky et al., 1980; Hager and Donehower, 1980; Majors and Varmus, 1980; Swanstrom et al., 1981). The RNAs which only anneal to cDNA_{5'} are probably generated by transcription of host cell sequences using a viral promoter supplied by an LTR positioned at the right or left ends of normal or abnormal proviruses. Two models for the origin of these RNAs are diagrammed in Figure 8A. Transcription could originate within the right LTR and directly proceed into host cell sequences, or could originate within the left LTR and proceed through the entire provirus into cell sequences. In the latter case, the RNA must then be processed (spliced) to remove most of the viral sequences. In the case of tumor LL 1, which lacked the left LTR, the RNA probably originated within the right LTR (Figure 8B). In other instances in which such RNA species have been observed, we have yet to determine which LTR initiated the transcripts.

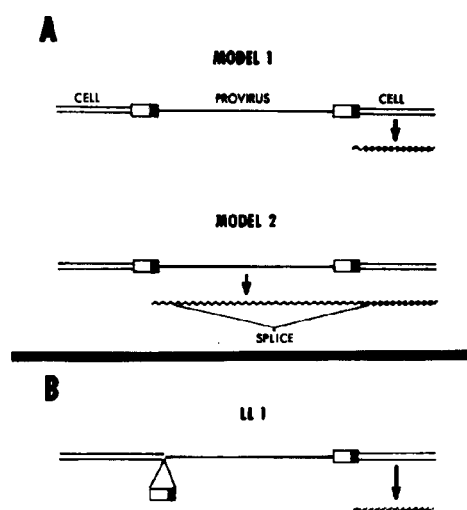


Figure 8. Possible Mechanisms for Generating Transcripts of Host Cell DNA from a Viral Promoter

(A) In the first model, transcription initiates in the right LTR and proceeds directly into flanking cell DNA. The second model postulates that transcription originates in the left LTR and reads through possible termination signals in the right LTR. The final transcript is formed by a processing event which removes most of the viral sequences.

(B) The structure of the provirus in tumor LL 1 from which the left LTR had been deleted. The probable origin of the RNA which annealed only to cDNA_{5'} is drawn below the provirus.

Are ALV Proviruses Integrated into Similar Regions of the Genome in Different Bursal Lymphomas?

Provirus-promoted transcription of host sequences represents a mechanism by which ALVs could exert a tumorigenic effect. Recent experiments of Cooper and his colleagues (1980) suggest that normal cell sequences taken out of the context of their normal flanking regions can exert transforming (oncogenic) effects. ALVs may subvert transcriptional control of certain cellular genes by inserting an upstream viral promoter; heightened or aberrant expression of such genes could conceivably extinguish growth control of B lymphocytes and produce leukosis. A similar mechanism might be employed by mammalian retroviruses such as MuLV, MMTV and bovine leukemia virus, which also induce clonal tumors after a long latent period (Steffen and Weinberg, 1978; Cohen et al., 1979; Cohen and Varmus, 1980; Jahner et al., 1980; Kettman et al., 1980).

One prediction of this model is that the proviruses present in different tumors will be integrated near a specific subset of cell genes capable of exerting oncogenic effects on the B lymphocyte. This does not imply that RAV-2 integrates into a small number of sites in the chicken genome. On the contrary, ample data suggest that retroviruses integrate into many sites in the host genome (Hughes et al., 1978; Steffen and Weinberg, 1978; Bacheler and Fan, 1979; Canaani and Aaronson, 1979; Cohen et al., 1979; Gilmer

and Parsons, 1979; Keshet et al., 1979; Ringold et al., 1979; van der Putten et al., 1979; Cohen and Varmus, 1980; Collins et al., 1980; Groner and Hynes, 1980; Hughes et al., 1981a; Jahner et al., 1980; Kettman et al., 1980; Majors and Varmus, 1981; Quintrell et al., 1980). We propose that RAV-2 infects many cells in the bursa, integrating at different sites in most cells. However, only a limited number of integration events may place the provirus in a position to initiate tumorous growth. This hypothesis could account for the clonal nature and lengthy latency of ALV-induced tumors.

After completing the experiments described here, we learned from Hayward and his colleagues that the ALV proviruses in the tumors described by Neel et al. (1981) were inserted near the cellular sequences related to the putative transforming gene of myelocytomatosis virus-29 (MC-29); moreover, some of the RNA species detected with cDNA₈ but not with cDNA_{rep} (Neel et al., 1981) appeared to anneal with cDNA specific for the transforming region of MC-29 (Hayward et al., 1981). We have therefore reexamined materials from our tumors with a probe derived from the same region of cloned MC-29 circular DNA (B. Vennstrom and J. M. Bishop, manuscript in preparation). All of our tumors, including LL 1–LL 4, contained proviruses closely linked to the cellular homolog of the MC-29 *onc* gene, confirming the results of Hayward et al. (1981). In some cases, the MC-29-specific sequences were located to the 3' side of the proviruses; in LL 2 and LL 3 the MC-29-specific region was positioned on the 5' side of the proviruses. The latter results are consistent with the restriction maps of the integration sites for proviruses in LL 1–LL 3 (Figure 5) and may be related to the absence of detectable viral RNA in LL 2 and LL 3.

Cooper and Neiman (1980) have recently found that NIH 3T3 fibroblasts can be transformed at high frequencies with DNA from ALV-induced bursal lymphomas. ALV proviral sequences (including sequences from the LTR) did not appear to be required for transformation of the NIH 3T3 cells. Further work is required to understand the relationship of these findings to those reported by us and by Neel et al. (1981).

Provirus in Metastases

Two of the tumors described in our report had metastasized to either the liver or spleen. The single abnormal provirus present in each metastatic growth was identical by restriction mapping to the provirus present in the primary bursal lymphoma. Thus provirus amplification, virus spread or further major alterations in the structure of the provirus are not required to confer metastatic potential on the tumor cell population residing in the bursa. Our findings are clearly inconsistent with the proposal by Neiman et al. (1980) that amplification of viral DNA is associated with metastatic potential.

Deletions May Reflect Selection against Viral Expression

The abnormal structure of the exogenous proviruses present in the tumors was a striking feature of our results. Eight out of 12 tumors contained only proviruses with significantly altered conformation. A ninth tumor contained both nondefective and defective proviruses. However, it seems improbable that the provirus must undergo a structural change to exert its oncogenic capacity. We and others (Neiman et al., 1980; Neel et al., 1981; Y. K. Fung and H.-J. Kung, personal communication) have observed tumors containing only apparently normal exogenous proviruses. A more likely possibility is that cells containing abnormal proviruses defective in expression of viral antigens are selected during the process of tumor progression by the host immune response to viral proteins, especially the *env* glycoprotein.

We have encountered other examples of proviral deletions similar to those described here, involving sequences near or at the left LTR, under conditions which may select against the expression of viral genes. By selecting for phenotypic revertants of an ASV-transformed rat cell (Varmus et al., 1981), mutants bearing deletions affecting the left end of an ASV provirus and eliminating expression of the viral *src* gene have been obtained (Majors et al., 1980). Hughes et al. (1981b) and Hayward et al. (1979) have described several deletions affecting the left ends of endogenous chicken proviruses; Hughes et al. (1981b) have proposed that these mutated proviruses may pose less of an evolutionary disadvantage to their host than intact proviruses, thus accounting for their prevalence.

Experimental Procedures

Source of Tumors

Eighty SPAFAS line 11 and 35 RPRL 151₈ × 7₂ day-old chicks were hatched and placed in plastic canopy isolators. Both lines of chickens are maintained under specific pathogen-free conditions and are free of common avian pathogens, particularly lymphoid leukosis viruses. At one week of age, heparinized blood was collected and packed cells were frozen for DNA analysis. Forty SPAFAS and 35 151₈ × 7₂ chickens were inoculated at one week of age with 10⁵ infectious units of a stock of Rous-associated virus-2 (RAV-2) propagated from plaque-purified material obtained from P. K. Vogt. The SPAFAS line 11 birds were maintained in isolators to 281 days of age. The inoculated birds were palpated for bursal tumors at least weekly from 140 to 240 days of age. When bursal enlargement was noticed by palpation, the birds were bled and killed. Portions of the bursa and any gross tumors were quick-frozen in liquid nitrogen. Portions of the brain, pancreas, muscle and blood were also saved. Portions of each tissue, except blood, were saved for DNA analysis and were also fixed in formalin and examined for histopathological evidence of tumor cells. Sex- and age-matched noninoculated control birds were killed, and corresponding material was taken for extraction of DNA and RNA. The incidence of leukosis in the infected SPAFAS chickens was 34% (11 of 32 survivors). The incidence of leukosis in the 151₈ × 7₂ chickens was 48.5% (16 of 34 survivors). Three 151₈ × 7₂ tumor-bearing birds were also killed for analysis. The presence of subgroup B virus in the bursa was determined as in Crittenden et al. (1979). Bursal tumors, spleen and muscle were also prepared from an addi-

tional group of $15\frac{1}{2} \times 7\frac{1}{2}$ chickens inoculated with RAV-1 (obtained from P. K. Vogt) one day after hatching.

Tumor Cell Lines

Three tumor cell lines derived from lymphomas induced by subgroup A ALV were kindly provided by H. Hihara. The isolation and maintenance of these lines has been described (Hihara et al., 1977). Briefly, 1104B-1 and BK 4484A were passaged by 1:10 dilution every 2-3 days into RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco), 10% tryptose-phosphate broth (TPB) and 5 μ g/ml gentamycin. 1104X-5 was passaged in the same manner, except that trypsin was used to harvest the cells.

The RAV-2-infected transplantable tumor line (LSCT-RP6) was provided by W. Okazaki (Okazaki et al., 1980). The tumor line was maintained by inoculation of 10^6 - 10^7 cells into the pectoral muscle of one-day-old $15\frac{1}{2} \times 7\frac{1}{2}$ chicks. A single-cell suspension was made from one such tumor by teasing it apart with needles; the cells were grown in RPMI 1640 supplemented with 10% FCS, 10% TPB and 5 μ g/ml gentamycin. This cell line was designated R2B. The 10th in vitro passage of these cells was tumorigenic when inoculated onto the pectoral muscle of one-day-old chicks (unpublished results of S. A. Courtneidge). All of the cell lines were grown in an atmosphere of 10% CO_2 and 7% O_2 in nitrogen.

Preparation and Cloning of Viral DNA

RAV-2 viral DNA was obtained from chick embryo fibroblasts 48 hr after infection with RAV-2 at a high multiplicity ($\text{moi} = 1 \text{ IU/cell}$). Unintegrated viral DNA was prepared by Hirt fractionation (Hirt, 1967) as described previously (DeLorbe et al., 1980). Supercoiled viral DNA was isolated from Hirt supernatant fractions by acid phenol extraction (Zasloff et al., 1978). Supercoiled RAV-2 DNA was digested with Sac I and cloned into bacteriophage λ gtWES- λ B as described by DeLorbe et al. (1980).

Preparation of High Molecular Weight DNA from Chicken Tissue

Frozen tissue (approximately 1 g) was minced with a razor blade and added to 10 ml of buffer containing 10 mM Tris-HCl, pH 8.1, 250 mM Na_2EDTA and 25% v/v glycerol. The tissue was dispersed by homogenization (one stroke) using a motor-driven (Talboys Engineering, Inc.) Dounce homogenizer. Protease K (Merck) (200 μ g/ml) and SDS (1%) were added, and the solution was incubated at 50°C for 3 hr, then extracted with phenol:chloroform (1:1) until the aqueous phase cleared. The solution was then extracted one time with chloroform, and the nucleic acid precipitated with two volumes of ethanol. Precipitated DNA was removed by spooling the fibers around a glass rod, drained and resuspended in TE (10 mM Tris-HCl, pH 8.1, 1 mM Na_2EDTA).

Preparation of High Molecular Weight DNA from Cell Lines

Cells were pelleted and washed twice with Tris-glucose (0.14 M NaCl, 5 mM KCl, 5 mM glucose, 25 mM Tris-HCl, pH 7.4). The cells were resuspended in TE buffer at a final concentration of approximately 10^7 /ml. The cells were lysed by adding Protease K (200 μ g/ml) and 1% SDS and incubating as described above. The solution was extracted with phenol:chloroform, precipitated and resuspended as described above.

Preparation of RNA from Chicken Tissue

Whole-cell RNA was prepared using a modification of the guanidinium thiocyanate procedure developed by Chirgwin (Ulrich et al., 1977) and described by Robertson and Varmus (1979).

Preparation of RNA from Tissue Culture Cells

We extracted RNA from whole cells as described previously (Weiss et al., 1977).

Analysis of Cellular DNA and Viral DNA with Restriction Endonucleases

DNA prepared as described above was cleaved with restriction endonucleases and fractionated by electrophoresis through agarose gels. The fractionated DNA was transferred to nitrocellulose mem-

branes for subsequent analysis with radioactive cDNAs (Southern, 1975). We have described the details of these procedures elsewhere (Shank et al., 1978).

Analysis of Viral RNAs

RNAs were fractionated through 1.2% agarose gels containing methyl mercury hydroxide, transferred to diazobenzyloxymethyl cellulose paper (Alwine et al., 1977) and hybridized sequentially to multiple radioactive cDNAs as described by Quintrell et al. (1980).

Molecular Hybridization in Solution

RNA was hybridized with radioactive cDNAs in solution as described previously (Leong et al., 1972). The percent hybridization was normalized to the maximum hybridization achieved using RNA from ALV-infected bursal lymphocytes derived from a bursal lymphoma (line BK 4484A; see above). The data was expressed as a function of Crt (mole-sec/l) corrected to standard conditions. The number of copies of RNA per cell was calculated as described in Spector et al. (1978b).

Preparation of Molecular Hybridization Probes

We have described previously the preparation and characterization of cDNA_{RAV} (Shank et al., 1978) and cDNA_{SR} (Tal et al., 1977). The Bam C probe and, in some cases, cDNA_{RAV} were prepared using restriction fragments derived from SR-A RSV cloned in bacteriophage λ gtWES- λ B and subcloned into pBR322 (DeLorbe et al., 1980). The appropriate restriction fragments were purified from pBR322 either by centrifugation through a sucrose gradient (DeLorbe et al., 1980) or by electrophoresis through low-melting (Seaplaque) agarose gels. The restriction fragments were localized in the gel by transillumination with ultraviolet light, and the appropriate region of the gel was removed and resuspended in a large volume, usually 20 ml, of STE (0.3 M NaCl, TE) plus 0.5% SDS and incubated at 68°C for 30 min. The solution was extracted with one volume of phenol, and the phenol phase was reextracted with STE. This solution was extracted three times with butanol:isopropyl alcohol (7:3) and the DNA was precipitated with two volumes of ethanol.

Probes were synthesized using these restriction fragments as templates as described by Shank et al. (1978), with the following changes: a denatured DNA restriction fragment was substituted for the RNA template, and a ratio of 50 μ g of calf thymus primers to 1 μ g of restriction fragment was employed.

cDNA_{RAV} was prepared by incubating 9 μ g of 70S viral RNA in a 300 μ l reaction mix, similar to that used in the preparation of cDNA_{SR}, except that oligo(dT) was omitted, the concentration of the unlabeled nucleotides was 25 μ M and 300 μ Cl of a ^{32}P -dCTP (2000-3000 Ci/mole, Amersham or NEN) was used. The reaction product was separated from unincorporated nucleotides by gel filtration with Sepharose G-50 and loaded on an 8% sequencing gel (Maxam and Gilbert, 1977). Bands representing cDNA_{RAV(100)} and cDNA_{RAV(70)} (Friedrich et al., 1977) were located by autoradiography and eluted from gel slices according to the procedure of Maxam and Gilbert (1977). cDNA_{RAV(100)} was used for analysis of cellular DNA and cDNA_{RAV(70)} for analysis of RNA.

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